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Development of an analytical method using reversed-phase HPLC-PDA for a semipurified extract of *Paullinia cupana* var. *sorbilis* (guaraná)

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ABSTRACT

The Neotropical plant 'guaraná' has been widely used in medicine, cosmetics, and industry because of its versatile biological activities. These effects are mainly attributed to the presence of polyphenols. An efficient, precise, and reliable method was developed for quantification of the polyphenols catechin and epicatechin in guaraná extract solution, using HPLC-PDA detection. The ideal conditions for the analysis of a semipurified extract of guaraná (EPA), using solutions of 0.05% TFA–water (phase A) and 0.05% TFA in acetonitrile:methanol (75:25, v v^{-1}) (phase B) as mobile phases were established. Gradient reversed-phase chromatography was performed using a guard cartridge (C18, 4.6 mm × 20 mm, 4 μm) and column (C18, 250 mm × 4.6 mm, 4 μm), flow of 0.5 mL min^{-1} and detection at 280 nm. The main validation parameters of the method were also determined. The method was linear over a range of 18.75–300 μg mL^{-1} for catechin and epicatechin, with detection limits of 0.70 and 0.88 μg mL^{-1} and quantification limits of 2.13 and 2.67 μg mL^{-1}, respectively. The method also showed consistent mean recoveries of 91.3 ± 3.8%, 2.14 RSD and 93.4 ± 3.1, 2.74 RSD of catechin and epicatechin respectively. The relative standard deviations were relatively low: intra-day (0.72% and 0.66% for catechin and epicatechin, respectively) and inter-day (0.93% and 0.75% for catechin and epicatechin, respectively). The semipurified extract showed catechin, epicatechin, and caffeine contents of 180.75, 278.87, and 300.87 μg mg^{-1}, respectively. The results demonstrated the efficiency, precision, accuracy, and robustness of the proposed method. The solutions remained stable for a sufficient time (one week) to complete the analytical process.

1. Introduction

The guaraná plant (*Paullinia cupana* var. *sorbilis* (Mart.) Ducke, Sapindaceae) is widely distributed in the Amazon region and also grows in northeastern Brazil, including the state of Bahia. Its seeds, used in popular medicine, contain large amounts of methylxanthines including caffeine, theophylin and theobromin, saponins, and polyphenols, especially tannins [1,2]. Guaraná extract is used as a stimulant of the central nervous system, in cases of physical and mental stress, and as an antidiarrheal, diuretic, and antineuralgic [1,3]. The antidepressive effect has been reported to be comparable to that of the tricyclic antidepressant imipramine, and with a beneficial effect on cognition, without altering locomotor activity [4–8]. Guaraná extract also shows low toxicity, with antioxidant and antiamnesiac effects [5,6,9–11], potential effect as a chemoprophylactic in carcinogenesis [9], and potential antibactericidal activity against *Streptococcus mutans*, a cause of bacterial dental plaque [3].

Chemical assay of a semipurified fraction of guaraná (EPA) showed the presence of caffeine, epicatechin, catechin, *ent*-epicatechin and procyanidins B1–B4, A2 and C1 [2,3]. This fraction showed an antidepressant effect on animals that received chronic treatment. This activity could not be related to the methylxanthins present, because when caffeine is tested in isolation, the effects differ from those of the EPA fraction. This suggests that the activity results from the presence of other constituents, and the condensed tannins may be the responsible agents; condensed tannins can cross the blood–brain barrier and act on the central nervous system [2,5,6,12]. Previous studies found that the EPA fraction of guaraná caused no toxicity in rats at the smallest dose evaluated (30 mg kg^{-1}) [13].

The potential for using guaraná in a wide range of medicinal applications justifies the interest in the quality control and standardization of its preparations. Capillary electrophoresis [14,15], mass spectrometry, and high-performance liquid chromatography (HPLC) [16,17] have been used to analyze the polyphenols, but the analytical procedures were complex, with long analysis times and dependent on the use of several polyphenols, analytical standards, and expensive reagents. Some analytical methods have employed HPLC to analyze *P. cupana*, but most of them describe the separation of methylxanthines [14,18–20]. Polyphenols, mainly tannins, have been isolated from other plants, but the method is often time-consuming (30–36 min [21]; 50 min [22]; and 55–106 min [23]).

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The aim of the present study was to develop and validate a reversed-phase HPLC-photodiode array (PDA) method for the separation and quantification of the catechin and epicatechin constituents in semipurified extract of guaraná. The main validation parameters of the method were also determined.

2. Experimental

2.1. Chemicals and reagents

Methanol and acetonitrile (J.T. Baker; HPLC grade), water filtered through a Milli-Q apparatus (Millipore), and trifluoroacetic acid (TFA) (J.T. Baker) were used as the mobile phase. Analytical-grade standards of catechin, epicatechin, and caffeine (Sigma) were used as external standards. Procyanidins B1 and B2 were isolated and identified by Ushirobira et al. [2] and Yamaguti-Sasaki et al. [3]. Acetone and ethyl acetate (Merck; analytical grade) were also used.

2.2. Apparatus

High performance liquid chromatography analyses were performed using a Thermo HPLC equipped with pumps and an integral degasser (Finnigan Surveyor LC Pump Plus), PDA spectrophotometric detector module (Finnigan Surveyor PDA Plus Detector), controller software (Chromquest) and autosampler (Finnigan Surveyor Autosampler Plus) equipped with a 10 μL loop and 10 μL injection. Chromatographic separation was accomplished using a Phenomenex® Synergi POLAR–RP 80A stainless-steel analytical column (250 mm × 4.6 mm, 4 μm) and a Phenomenex® C18 guard cartridge system (4 μm, 4.6 mm × 20 mm). The mobile phase used was a gradient system of 0.05% TFA–water (phase A) and 0.05% TFA- acetonitrile:methanol (75:25, v v^{-1}) (phase B), previously degassed using an ultrasonic bath. The gradient system was established and demonstrated in Section 3. Gradient separation was performed at a flow rate of 0.5 mL min^{-1}. Another HPLC analysis was carried out using a different column, a Waters X Bridge™ C18 (100 mm × 4.6 mm, 5 μm) and a Waters X Bridge™ C18 guard cartridge system (5 μm, 4.6 mm × 20 mm).

For the interlaboratory HPLC assay, a different apparatus was used, a Gilson HPLC system consisting of a Model 321 pump, a Model 156 variable-wavelength UV/Vis detector, a Rheodyne manual injection valve with a 10 μL loop, Model 184 degasser, a Model 831 thermostatted column compartment, and Unipoint LC system software.

2.3. Preparation of the EPA extractive solution

Guaraná samples obtained in the municipality of Alta Floresta, state of Mato Grosso, Brazil, were used to prepare the acetone:water (70:30) extractive solution (ES), by turbo extraction (Ultra-Turrax UTC115KT, IKA Works, Wilmington, NC, USA). After the organic solvent was removed, the remaining solid material was lyophilized (EBPC; patent pending PI0006638-9). The EBPC (crude extract) was partitioned with ethyl acetate, resulting in an ethyl-acetate fraction (EPA) [4,13]. The EPA was extracted with solid-phase extraction (SPE). A 2.00 mg portion of EPA was diluted in 1 mL of 20% methanol and was passed through the SPE cartridge and diluted in 25 mL of 20% methanol. A 10 μL aliquot was analyzed by HPLC.

2.4. Method validation

For validation of the analytical method, the guidelines established by the ICH (International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) and by Brazilian regulation RE 899/2003 of the National Health Surveillance Agency (ANVISA) were employed [24,25].

2.4.1. Linearity

Linearity was determined by the calibration curves obtained from the HPLC analyses of the standard solutions of catechin and epicatechin. The range (interval between the upper and lower concentrations of analyte in the sample) of the appropriate amount of samples was determined. The slope and other statistics of the calibration curves were calculated by linear regression and analysis of variance (ANOVA).

The catechin and epicatechin standards were dissolved in 20% methanol to give concentrations of 18.75, 37.5, 75.0, 150, and 300 μg mL^{-1}. The solutions were filtered through an FHLP01300 20 μm membrane filter (Millipore). Evaluation of each point was conducted in five replicates, and the calibration curve was fitted by linear regression.

2.4.2. Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) and the slope (S) of the calibration curve based on Eqs. (1) and (2).

$$\mathrm{LOD} = \frac{3.3 \times \mathrm{SD}}{S} \quad (1)$$

$$\mathrm{LOQ}\frac{10 \times \mathrm{SD}}{S} \quad (2)$$

2.4.3. Precision

The precision of the method was determined following ICH guidelines. Precision was evaluated at three levels: repeatability, intermediate precision, and reproducibility. The standard deviation (SD) and relative standard deviation (RSD) of six injections at 100% of the test concentration were evaluated and analyzed intra-day and inter-day, and with different analysts and different apparatus.

2.4.4. Accuracy

The accuracy was determined by recovery analyses, adding measured amounts of catechin (100, 50, and 25 μg mL^{-1}) and epicatechin (100, 50, and 25 μg mL^{-1}) to EPA extractive solution samples. The recovery experiments were performed in triplicate. The recovery data were determined by dividing the value obtained for the sample prepared with the added standard, by the amount added, and then multiplying by 100% [24].

2.4.5. Robustness

The robustness was determined for variations in flow rates, for 0.495 mL min^{-1} and 0.505 mL min^{-1}. The Tukey test of ANOVA was performed to evaluate whether the flow variations altered the results of the HPLC analysis.

2.4.6. Stability

The stability of the EPA extractive solutions was determined over a period of four weeks. A 2.00 mg portion of EPA was diluted in 1 mL of 20% methanol. This solution was passed through the SPE cartridge and diluted in 25 mL of 20% methanol. The samples were stored at room temperature, exposed to light. A 10 μL aliquot was analyzed by HPLC.

2.5. EPA extractive solution quantification

The catechin, epicatechin and caffeine calibration curves were utilized to quantify the EPA extractive solutions. The EPA extractive solutions were analyzed by HPLC in six replicates. The catechin, epicatechin, and caffeine peaks were quantified by linear regression of the standards.